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Molecular crowding affects diffusion and binding of nuclear proteins in heterochromatin and reveals the fractal organization of chromatin

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1st Editorial Decision 02 September 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from their comments the referees express interest in the study and find that it will form the basis for future work on the dynamics of nuclear proteins and organization. They all raise a number of issues that should be incorporated into the text of a revised version of the manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Bancaud et al. investigate molecular crowding inside of nuclei. They use fluorescent dextrans or GFPs of different sizes and measure 1.) their relative intensities and 2.) their diffusion constants in euchromatin, heterochromatin and nucleoli. They find evidence that in both heterochromatin and nucleoli, molecules diffuse more slowly (and anomalously) and are at reduced concentrations relative to euchromatin. These features are predicted for environments where there is molecular crowding. Using photoactivation microscopy, they also investigate the binding properties of three generic chromatin binding proteins and find that the binding within heterochromatin (but not euchromatin) is not well explained by conventional reaction-diffusion models. They propose a fractal model for binding kinetics in heterochromatin and show that this leads to improved fits of the photoactivation data in heterochromatin, and that it can also explain the observations of molecular crowding for diffusible molecules.

This is a fascinating paper that has the potential to open up a new area of exploration in nuclear cell biology. The authors have synthesized a number of disparate biophysical studies to generate an interesting model of how chromatin architecture can give rise to anomalous diffusion and binding behavior inside of a cell nucleus. They suggest that the distribution of chromatin is fractal. If so, this would have important consequences for a variety of nuclear processes, some of which the authors consider in their Discussion.

Despite the potentially important insights, the paper is at the same time dangerous in that it is written with such certainty. In fact, there are many reasons to be quite cautious about the conclusions of the manuscript. Perhaps the simplest is that to really establish that chromatin is a fractal, one would like to visualize chromatin structure and see evidence of the self similarity at different spatial scales. The authors instead infer this from fitting photoactivation data and measuring diffusion of quantum dots. This is an interesting start, but not definitive.

Another reason for caution is that analysis of live-cell data is still a comparatively new field. Although the authors find evidence for anomalous diffusion of GFP by FCS and cite a few other studies that have also observed this, there are still other studies which have been able to fit GFP FCS data with a conventional diffusion model (1-4). It remains a mystery why some groups observe anomalous diffusion and others do not. This discrepancy raises general questions about the field of FCS measurements in live cells.

The authors observe a relatively small plateau in their photoactivation data within heterochromatin, and this plays a key role in developing their model for fractal binding kinetics. It seems likely that other explanations could also be developed for this plateau, for example a second binding state that arises in heterochromatin. Alternatively, the photoactivation will have some extent in the axial direction and probably also includes therefore some euchromatin which might contribute a second component to the curve. Even assuming a fractal model for heterochromatin, it is not clear what the real distribution of binding sites might be. Quite likely they are not randomly distributed as the authors assume. Also related to the fractal kinetics model is the question of how the results would change if molecular crowding of co-solutes was also introduced into the model, in addition to the fractal structure of chromatin.

I do not think the authors necessarily need to address all of the preceding concerns by performing additional experiments. I raise these issues only to illustrate some of the many uncertainties which can affect their analysis. Rather what I think the authors should do is drastically tone down the certainty of their conclusions. In my view, this manuscript has constructed a very interesting model that is reasonably consistent with the experimental data presented. If they are even partially correct, this manuscript may help generate years of work on these questions by many other groups. Thus the current manuscript is an exploratory study, not the definitive work on this question. The authors should re-write the paper in a much more modest way that indicates at various points where uncertainties remain and how alternative explanations need to be considered in future studies.

Some detailed points:

Eq. 2 p 14. I could not find this equation in the citation (Kopelman, 1988).

P 21. Eq. 6 does not follow from Eq. 5. If Eq. 5 is written with an equal sign and a proportionality constant C, then log C enters as an additive constant when taking the log of both sides of Eq. 5.

P 22. The calculation of the fractal dimension is obtained by dividing the jump histogram obtained at two different frame rates to eliminate the factor P0. This assumes that P0 is the same for both experiments, whereas it is not clear that it should be. The probability density function must integrate to one in both cases and this might lead to different P0s.

P 23 The citation of Wu and Berland does not seem to connect with the definition of the autocorrelation function given, since Wu and Berland would argue that D would be time dependent, whereas the D's reported in the manuscript do not seem to be time dependent.

P 24, Fig. 1 legend. The sentence starting with "In the left plot" should be "In the right plot" ?

P 27, Fig. 4 legend. The labels of the panels are incorrect.

Supplemental Materials: Euchromatin is modeled with simple diffusion and conventional binding kinetics. Is this correct? Why does this work in euchromatin and give a good fit?

Supplemental Materials: The authors say that their simulations are implemented as in Schnell and Turner (2004). It seems however that the authors consider both the binding sites and the barriers as immobile, while this is not done in the cited paper.

Supplemental Materials: To address the singularity at zero, the authors introduce their own equation for fractal binding (Eq. S3). However, their equation is very similar to the Zipf-Mandelbrot distribution described in Schnell and Turner (2004). It seems it would be better to use this distribution and simply state in the main text of the paper that they are using a form of fractal kinetics given by Zipf-Mandelbrot.

Supplemental materials: Physics laws for fractal models. Most of the cited equations are lacking references.

References

1. Chen Y, M¸ller JD, Ruan Q, Gratton E. Molecular brightness characterization of EGFP in vivo by fluorescence fluctuation spectroscopy. Biophys J. 2002.

2. Wang Z, Shah JV, Chen Z, Sun CH, Berns MW. Fluorescence correlation spectroscopy investigation of a GFP mutant-enhanced cyan fluorescent protein and its tubulin fusion in living cells with two-photon excitation. J Biomed Opt. 2004.

3. Bhattacharya D, Mazumder A, Miriam SA, Shivashankar GV. EGFP-tagged core and linker histones diffuse via distinct mechanisms within living cells. Biophys J. 2006.

4. Renz, M., Langowski, J. Dynamics of the CapG actin-binding protein in the cell nucleus studied by FRAP and FCS. Chrom. Res. 2008.

Referee #2 (Remarks to the Author):

The authors investigate the influence of molecular crowding on diffusive and binding properties of macromolecules in euchromatin-compared to heterochromatin of living cells. The first part of the manuscript establishes the non-homogeneous distribution of crowding effects between euchromatin and heterochromatin, such as the size-dependent and disproportionate exclusion of tracer molecules and the retardation of diffusional motion within an FCS-focus. The second part addresses the observation of short-lived caging of endogenous chromatin binding proteins in heterochromatin and its interpretation in terms of a fractal model of chromatin organization.

I like to direct the following remarks to the authors:

1. Presentation of 'molecular crowding' appears to me too cryptic.
1.1. It might be illuminative to many readers, if the steric impact which makes the difference between 'concentration' and 'crowding' was explained, respectively the impact of particle-geometry for the difference between the volume occupied by cosolutes and the volume excluded to a tracer.
1.2. It should become clear, that 'crowding' is always there in a system of macromolecular content irrespective of amounts and composition. Rather, it is the degree of crowding, which matters. Formulations like '...inducing heterochromatin produces molecular crowding...' (Results p. 12) are thus misleading.

2. Introduction, p.3, end of first paragraph:
Macromolecular crowding is not directly challenged by osmotic agents: Hancock used inert macromolecules like dextran and polyethylene glycol to reconstitute nucleoli and PML bodies in vitro; Richter et al. used hypertonic stress to increase the crowding of endogenous macromolecules by the extraction of water from living cells.

3. Introduction p. 4 line 9
Volume exclusion does not 'favor protein configurations associated with minimal entropy'. Rather, aggregates will form if the accompanied loss of entropy is counterbalanced by a gain of entropy somewhere else in the system. This subtle difference not only prevents conflict with the second law of thermodynamics, but clarifies, why compaction is not necessarily driven to a minimum.

4. Results, p.6 and Fig. 1b. Relative availability of heterochtomatin/ euchromatin for different sized tracers:
4.1. Compaction-ratios for measured Heterochromatin:Euchromatin were 6:1. In the introduction (page 3), a ratio of 4:1 is cited. In the discussion (page 17) a ratio 2:1 was used for model calculations. How is this discrepancy explained?
4.2 How representative could sites be chosen to measure euchromatin?

5. Suppl. 2b:
How is the observed variability of nucleoplasmic-to-cytoplasmic concentration of tracers to be explained?

6. Results Fig 5a and discussion p13 line 14
The behavior of RCC1 does not fit well with the fractal model. This is explained by a change of fractal dimension with time. Why does this not apply to the other two, H1.1 and H1-t?

7. Discussion: p. 15, second paragraph
Though diffusional kinetics to fill available space was identical for a given tracer everywhere in the nucleus (shown with PA-experiments), the residence times measured by FCS did increase with the crowding-status. Is this discrepancy of motional behavior determined by either PA or FCS attributable to the differences in the available volumes in euchromatin and heterochromatin, which were not corrected for in the calculation of the FCS-auto-correlation function in contrast to the adequate normalization of PA-experiments?

8. Discussion p. 17/18
'...we predict, that heterochromatin exclusion relative to euchromatin should be 2 fold, in agreement with our measurement...'
The prediction precludes a 2-fold density-difference between eu- and heterochromatin. In contrast, according to Fig.1, measurements were based on 6-fold densitiy-ratios. Since accessible volume fractions disproportionally decrease with increasing volume occupancies of crowders, the apparent agreement needs more explication.

List of minor remarks:

- Results, p6 line 10: A very similar study was performed by Gorisch et al 2003 and should be cited together with Handwerger.

- Results p.6 line 11 and Fig. 1:The definition of 'exclusion' as the relative accessibility between two compartments is misleading, since tracers will become excluded from both compartments to some extent. 'Relative exclusion' may be more informative.

- Results, p.7, line 10: 'Fig 4b' should read Fig 2b

- Suppl. 2b: A line-scan through the cells of the third lane could help to appreciate the relative differences of GFP-10 expression in the three examples.

- Suppl. S2c and d: The four tracer-sizes (mGFP through mGFP-10) in the four cumulated graphs should be distinguished, e.g. using color coding for the measure points as in Fig. 4b, to visualize the influence of tracer size separated from effects of the nucleoplasm (in c) and euchromatin (in d).

- Results p. 9,l.4 Enhancement of binding rates is a possible but not necessary consequence of crowding; it depends on the reaction (e.g. Minton 2001).

- Suppl. S3 a: 'triangles' are squares.

- Suppl. S3 c: How is the 'compensation for PAGFP photophysics' performed?

- Suppl. S3 c: The plateau at short times scales is not readily appreciable, given the ups and downs throughout the entire curve progression. Could it be specified on the graph e.g. using arrows?

- Suppl. S3 d: Explain 'PDE'.

- Suppl. S3 e: The fit for heterochromatin (orange) not only is poor for short timescales. A smoothed plot of residuals probably would be more informative than the enlarged view of the short-time regime.

-Suppl. S3 f: 'Heterochromatin dynamics...' should probably read like 'dynamics of tracers within heterochromatin'.

- Results p.9, lower part: 'Fig. 1c' is Fig. 3c.

- Fig 4: labels d through f are out of scale.

- p.15, first paragraph: 'We thus propose molecular crowding as force that promotes and stabilizes the self-organization of nuclear compartments.' This has been proposed by many others before (e.g. Herzfeld 1996, Marenduzzo et al. 2006, Iborra 2007, Richter et al. 2008) and should be taken into account with this statement.

Referee #3 (Remarks to the Author):

In their paper Bancaud et al. present a comprehensive approach to characterizing the effects of crowding due to chromatin organanization in vivo. They report several different experimental studies, i.e. PA, FCS and single particle tracking, to quantify the main effects related to crowding, i.e. excluded volume, slower diffusion and enhanced binding rates. Furthermore, they unify the results of these experiments in a single model in which chromatin organization is described in terms of fractal dimensions.

The experiments seem well designed for the tested models and performed at a high level. Though not all experiments seem original, it is definitely valuable that all were done in the same laboratory and where possible with the same cell lines and probes. The true value of this paper is in the integration of all methods and in the original way of testing and describing crowding effects in vivo. This is an important issue and has been relatively ignored before. That data and the interpretation in the results section appear sound though the use of the residence time for estimation of the size of the mEGFP probe may be less solid. If the FCS signal consists of a mixture of different species, this might also result in a broadened and shallower correlation curve. However, the anomaly parameter appears independent of size giving credit to this interpretation.

In my opinion the paper presents novel ideas and has the potential to have a large impact on the field. I can highly recommend this paper for publication.

Textual remarks:
Page 8, line 9 states a diffusion coefficient of 9.2 +/- 1.0 um2/s in euchromatin while in figure 2b it says 10.5

Figure caption S5 e-f: remove repeated 'to chromatin'

Figure caption S5 g: insert ... scale depends on 'the' tracking accuracy

1st Revision - authors' response 07 October 2009

Referee #1
We are grateful to referee #1 for her/his critical reading of our work, and for her/his constructive comments in relationship with several contributions of the field.

Bancaud et al. investigate molecular crowding inside of nuclei. They use fluorescent dextrans or GFPs of different sizes and measure 1.) their relative intensities and 2.) their diffusion constants in euchromatin, heterochromatin and nucleoli. They find evidence that in both heterochromatin and nucleoli, molecules diffuse more slowly (and anomalously) and are at reduced concentrations relative to euchromatin. These features are predicted for environments where there is molecular crowding. Using photoactivation microscopy, they also investigate the binding properties of three generic chromatin binding proteins and find that the binding within heterochromatin (but not euchromatin) is not well explained by conventional reaction-diffusion models. They propose a fractal model for binding kinetics in heterochromatin and show that this leads to improved fits of the photoactivation data in heterochromatin, and that it can also explain the observations of molecular crowding for diffusible molecules.

This is a fascinating paper that has the potential to open up a new area of exploration in nuclear cell biology. The authors have synthesized a number of disparate biophysical studies to generate an interesting model of how chromatin architecture can give rise to anomalous diffusion and binding behavior inside of a cell nucleus. They suggest that the distribution of chromatin is fractal. If so, this would have important consequences for a variety of nuclear processes, some of which the authors consider in their Discussion.

Despite the potentially important insights, the paper is at the same time dangerous in that it is written with such certainty. In fact, there are many reasons to be quite cautious about the conclusions of the manuscript. Perhaps the simplest is that to really establish that chromatin is a fractal, one would like to visualize chromatin structure and see evidence of the self similarity at different spatial scales. The authors instead infer this from fitting photoactivation data and measuring diffusion of quantum dots. This is an interesting start, but not definitive.

We acknowledge that the fractality of chromatin is an interpretation based on the analysis of our data, and that we have do not provide a direct visualization of its fractal structure (visualizing chromatin topology under physiological conditions at high resolution is currently not possible by any method we are aware of). However, we provide three independent lines of evidence for the fractality of chromatin architecture based on FCS, SPT and PA. Furthermore, we emphasize the consistency of our model showing that all the rheological measurements that we performed can be interpreted with a minimal number of structural parameters. Therefore, our working model of a fractal organization, consistent with observations by neutron scattering on isolated nuclei (Lebedev, FEBS Lett, 2005), is strengthened by an ensemble of converging arguments. We have made it explicit in the revised manuscript that this is a working model (see below).

Another reason for caution is that analysis of live-cell data is still a comparatively new field. Although the authors find evidence for anomalous diffusion of GFP by FCS and cite a few other studies that have also observed this, there are still other studies which have been able to fit GFP FCS data with a conventional diffusion model (1-4). It remains a mystery why some groups observe anomalous diffusion and others do not. This discrepancy raises general questions about the field of FCS measurements in live cells.

The referee raises an interesting point. Before going into more details, we would like to point out that the acronym FCS in this remark stands for different techniques. First, 2-photon FCS is used in references (1,2), whereas standard single photon FCS is the technique of references (3,4). Moreover, the working model to fit autocorrelation curves varies in the papers mentioned by the referee. In the single photon FCS studies (3,4), the authors assume that GFP photophysics determines the shape of the auto-correlation function in the short time regime, but they do not necessarily document the fitting parameters associated with this model (see 3). Although we acknowledge these discrepancies in the field of FCS, we observe anomalous diffusion for the monomer, dimer, pentamer and decamer of mEGFP, i.e. for a family of freely diffusing tracers. In addition, we strengthen our argument on anomalous diffusion by showing the very similar rheological response of quantum dots as inferred from single particle tracking. Notably, anomalous diffusion is also consistent with previous studies using single particle tracking of fluorescent nanoparticles (Tseng, JCS, 2004), or fluorescently labelled proteins (Kues, BJ, 2001).

The authors observe a relatively small plateau in their photoactivation data within heterochromatin, and this plays a key role in developing their model for fractal binding kinetics. It seems likely that other explanations could also be developed for this plateau, for example a second binding state that arises in heterochromatin. Alternatively, the photoactivation will have some extent in the axial direction and probably also includes therefore some euchromatin which might contribute a second component to the curve. Even assuming a fractal model for heterochromatin, it is not clear what the real distribution of binding sites might be. Quite likely they are not randomly distributed as the authors assume. Also related to the fractal kinetics model is the question of how the results would change if molecular crowding of co-solutes was also introduced into the model, in addition to the fractal structure of chromatin.

The diffusion-reaction scheme that fits euchromatin dynamics fails to reproduce heterochromatin responses. We propose to fit this data with a fractal kinetics model, and we performed many controls to validate this approach, which are described in Fig. S4. The referee raises two issues.
First, the size along the equatorial axis of the photoactivated region is larger than heterochromatin foci (Fig. 3a), and the focal depth that is scanned during fluorescence redistribution is 1 m, i.e. comparable with the size of heterochromatin foci. We agree that some euchromatin signal from the equatorial direction may be sampled in our experiments. However, we demonstrate that euchromatin kinetics are faster than heterochromatin, and the contribution of euchromatin in heterochromatin relaxations can only speed up the actual kinetics, and would thus lead us to underestimate the magnitude of the heterochromatin specific plateau at the early time points. In addition, we implemented 3D models with Berkeley Madonna that reproduce the typical size of heterochromatin foci and photoactivated regions (Fig. S4), and we clearly demonstrate that standard 3D diffusion-reaction schemes provide poor fits to our data.
Second, the referee raises a point about the exact distribution of binding sites. In our models, the binding site density map is always inferred from the steady state distribution of chromatin interacting proteins prior to photoactivation, which quantitatively assays the density of binding sites independently of the number of components in the reaction mechanism. We therefore do not quite understand the remark "Quite likely they are not randomly distributed as the authors assume." because we take into account the non-random steady state distribution and our model assumes non-randomly distributed binding sites, i.e. a fractal architecture.

I do not think the authors necessarily need to address all of the preceding concerns by performing additional experiments. I raise these issues only to illustrate some of the many uncertainties which can affect their analysis. Rather what I think the authors should do is drastically tone down the certainty of their conclusions. In my view, this manuscript has

constructed a very interesting model that is reasonably consistent with the experimental data presented. If they are even partially correct, this manuscript may help generate years of work on these questions by many other groups. Thus the current manuscript is an exploratory study, not the definitive work on this question. The authors should re-write the paper in a much more modest way that indicates at various points where uncertainties remain and how alternative explanations need to be considered in future studies.

We took into account the referee's advice by making it even more clear that the fractality of chromatin is the best working model at the following passages:
p.12: "chromatin architecture ~~appears to be fractal~~ is consistent with a fractal model at length scales smaller than ~100 nm, leading to a size independent obstruction for the vast majority of proteins or nucleoprotein complexes diffusing in the nucleus."

p.13: "Our results show that the dynamics of chromatin interacting proteins ~~exhibit~~ are consistent with fractal binding kinetics."
three independent lines of evidence therefore ~~strongly~~ support a fractal working model of chromatin architecture as the main crowding agent in the nucleoplasm.

p.14: "Thus, the fractal architecture of chromatin appears to be modulated in nuclear compartments~~. The~~ with a fractal dimension that is larger in eu- than in heterochromatin"

p.16: "Chromatin architecture ~~is~~ is consistent with a fractal model, heterochromatin fills space more compactly than euchromatin"

Some detailed points:

Eq. 2 p 14. I could not find this equation in the citation (Kopelman, 1988).

We apologize for this referencing error and have corrected the reference to Kopelman, R. (1986) Rate Processes on Fractals: Theory, Simulations and Experiments. Journal of Statistical Physics, 42, 185-200, where the equation can be found in Eq. 12a.

P 21. Eq. 6 does not follow from Eq. 5. If Eq. 5 is written with an equal sign and a proportionality constant C, then lo g C enters as an additive constant when taking the log of both sides of Eq. 5.

We agree with the referee that there are some simplifications when going from Eq. 5 to Eq. 6, as there is a proportionality constant in Eq. 5 that depends on the dimensionality of the problem. However, because we are interested in the temporal dependence of Eq. 5, we may disregard this constant that does not interfere with the measurement of the anomalous parameter. Notably, this plot has been extensively used by specialists of the field including M. J. Saxton, see e.g. (Saxton, Biophhys, 1994).

P 22. The calculation of the fractal dimension is obtained by dividing the jump histogram obtained at two different frame rates to eliminate the factor P0. This assumes that P0 is the same for both experiments, whereas it is not clear that it should be. The probability density function must integrate to one in both cases and this might lead to different P0s.

The fractal dimension is obtained without changing the frame rate, and using the same particle trajectory. Our computation is obtained from two step histograms calculated for time intervals of 1.9 ms and 7.8 ms, i.e. with an interval of four images.
We tried to clarify this point by rewriting:
Materials and methods, section Imaging and Photoactivation:
"To remove P_0, we computed the probability distribution function of individual trajectories and calculated the ratio at two time points"

P 23 The citation of Wu and Berland does not seem to connect with the definition of the autocorrelation function given, since Wu and Berland would argue that D would be time dependent, whereas the D's reported in the manuscript do not seem to be time dependent.

In our study, the diffusion coefficient clearly depends on time, as observed with SPT experiments (Fig. 4d). In FCS, the observation volume is defined by the optical settings of the microscope, and an apparent diffusion coefficient is measured that depends on the observation lengthscale. Wu and Berland nicely demonstrate that the anomalous coefficient inferred from SPT and FCS is similar, as observed in our experiments.

P 24, Fig. 1 legend. The sentence starting with "In the left plot" should be "In the right plot"?

This point has been corrected in the revised version of the manuscript.

P 27, Fig. 4 legend. The labels of the panels are incorrect.

We corrected this mistake in the updated version.

Supplemental Materials: Euchromatin is modeled with simple diffusion and conventional binding kinetics. Is this correct? Why does this work in euchromatin and give a good fit?

The analysis of euchromatin redistribution kinetics involves a minimal diffusionreaction mechanism, as described in supplementary equation S1. This model was already shown to be sufficient for fit the response of many chromatin interacting proteins (see Beaudouin et al. 2006, and Sprague et al. 2005 & 2007). Because transient interactions appear to be common for nuclear proteins, and the temporal and spatial resolution of FRAP tools is limited to 10 ms and 1 m, diffusion appears to be the limiting factor of the redistribution, and a simple model is sufficient to describe such kinetics.

Supplemental Materials: The authors say that their simulations are implemented as in Schnell and Turner (2004). It seems however that the authors consider both the binding sites and the barriers as immobile, while this is not done in the cited paper.

We agree with the remark of the referee, but we used the Schnell and Turner paper for reference in our model. We thus changed the text of the Supplementary Material for:
"2D molecular dynamics simulations were implemented using a method inspired from (Schnell and Turner, 2004), yet simplified:"

Supplemental Materials: To address the singularity at zero, the authors introduce their own equation for fractal binding (Eq. S3). However, their equation is very similar to the Zipf-Mandelbrot distribution described in Schnell and Turner (2004). It seems it would be better to use this distribution and simply state in the main text of the paper that they are using a form of fractal kinetics given by Zipf-Mandelbrot.

We mentioned the use of Zipf-Mandelbrot distributions in the materials and methods section:
"The detailed implementation of normal and fractal kinetics based on Zipf-Mandelbrot distributions (Schnell and Turner, 2004) with the Berkeley Madonna solver is provided in Supplementary Material."

Supplemental materials: Physics laws for fractal models. Most of the cited equations are lacking references.

This omission has been corrected, and the Neale and Nader model is referenced.

References

1. Chen Y, Müller JD, Ruan Q, Gratton E. Molecular brightness characterization of EGFP in vivo by fluorescence fluctuation spectroscopy. Biophys J. 2002.

2. Wang Z, Shah JV, Chen Z, Sun CH, Berns MW. Fluorescence correlation spectroscopy investigation of a GFP mutant-enhanced cyan fluorescent protein and its tubulin fusion in living cells with two-photon excitation. J Biomed Opt. 2004.

3. Bhattacharya D, Mazumder A, Miriam SA, Shivashankar GV. EGFP-tagged core and linker histones diffuse via distinct mechanisms within living cells. Biophys J. 2006.

4. Renz, M., Langowski, J. Dynamics of the CapG actin-binding protein in the cell nucleus studied by FRAP and FCS. Chrom. Res. 2008.

Referee #2

We thank the referee for her/his critical reading of the manuscript, and her/his good suggestions that improve the readability of our work.

The authors investigate the influence of molecular crowding on diffusive and binding properties of macromolecules in euchromatin-compared to heterochromatin of living cells. The first part of the manuscript establishes the non-homogeneous distribution of crowding effects between euchromatin and heterochromatin, such as the size-dependent and disproportionate exclusion of tracer molecules and the retardation of diffusional motion within an FCS-focus. The second part addresses the observation of short-lived caging of endogenous chromatin binding proteins in heterochromatin and its interpretation in terms of a fractal model of chromatin organization.

I like to direct the following remarks to the authors:

1. Presentation of 'molecular crowding' appears to me too cryptic.

1.1. It might be illuminative to many readers, if the steric impact which makes the difference between 'concentration' and 'crowding' was explained, respectively the impact of particlegeometry for the difference between the volume occupied by cosolutes and the volume excluded to a tracer.

We changed our introductory sentence on molecular crowding to improve the readability:
"Classical molecular crowding studies, reviewed in (Minton, 1995; Zimmerman and Minton, 1993), investigate in vitro biophysical and biochemical consequences of the presence of large amounts of inert cosolutes that reduce the available volume by steric interaction in a reaction medium."

1.2. It should become clear, that 'crowding' is always there in a system of macromolecular content irrespective of amounts and composition. Rather, it is the degree of crowding, which matters. Formulations like '...inducing heterochromatin produces molecular crowding...' (Results p. 12) are thus misleading.

We fully agree with this statement, and thus changed the sentence on p. 12:
"This is confirmed by our observation that inducing heterochromatin formation increases the degree of molecular crowding"

2. Introduction, p.3, end of first paragraph:
Macromolecular crowding is not directly challenged by osmotic agents: Hancock used inert macromolecules like dextran and polyethylene glycol to reconstitute nucleoli and PML bodies

in vitro; Richter et al. used hypertonic stress to increase the crowding of endogenous macromolecules by the extraction of water from living cells.

The remark is valid, and we corrected the introduction:
"As an alternative and not mutually exclusive model, macromolecular crowding has been suggested as a general driving force for self-organization of nuclear compartments based on the osmotic manipulation of nucleoli in isolated nuclei (Hancock, 2004), and the use of hypertonic stress in intact cells (Richter et al., 2007)."

3. Introduction p. 4 line 9
Volume exclusion does not 'favor protein configurations associated with minimal entropy'. Rather, aggregates will form if the accompanied loss of entropy is counterbalanced by a gain of entropy somewhere else in the system. This subtle difference not only prevents conflict with the second law of thermodynamics, but clarifies, why compaction is not necessarily driven to a minimum.

We fully agree with the referee on this imprecision, which can be misleading especially regarding the second principle of thermodynamics. However, we believe that the details of this discussion are somewhat beyond the scope of the introduction, particularly for a biological readership.
Rather than going into this subtle discussion, we propose the following sentence, which is less affirmative:
"favor protein configurations associated with a reduction of entropy"

4. Results, p.6 and Fig. 1b. Relative availability of heterochtomatin/ euchromatin for different sized tracers:

We agree with the referee that "availability" is an interesting alternative term to "exclusion". However, heterochromatin exclusion is a common expression in the field of chromatin biochemistry, and we prefer to keep this phrasing that will be better understood by the biology community. In line with the second minor remark of the referee, we agree to use relative exclusion that seems to be the most appropriate phrasing.
Our textual changes follow:
p.6: "We quantified the relative exclusion, defined by the nucleolar to nucleoplasmic concentration ratio. Relative exclusion was found to be size dependent and to increase with probe size"
caption of fig. 1: "The right panel shows nucleolar vs. nucleoplasmic relative exclusion of four different inert probes in NRK."

4.1. Compaction-ratios for measured Heterochromatin:Euchromatin were 6:1. In the introduction (page 3), a ratio of 4:1 is cited. In the discussion (page 17) a ratio 2:1 was used for model calculations. How is this discrepancy explained?
4.2 How representative could sites be chosen to measure euchromatin?

In fig. 1, we use a Hoechst to stain heterochromatin foci. This marker is excited in the UV spectrum, two markers additional markers (e.g. GFP and RFP) can be used to monitor heterochromatin vs. euchromatin relative exclusion. However, Hoechst exhibits a sequence preference for AT rich regions, which are overrepresented in heterochromatin, and it is not considered as an accurate chromatin reporter. Nevertheless, there is no contradiction with our conclusions from Fig. 1 because we show that size-dependent exclusion occurs in heterochromatin, and that even large tracers are not completely excluded from this compartment. H2B-mRFP, which is a constitutive nucleosome component, is considered to be more reliable for heterochromatin density maps. Its use enabled us to monitor that H1.1 and H2B accumulation in heterochromatin is exactly similar (data not shown). Thus, faithful chromatin density pictures can be found in Fig. 3a. In our hands, heterochromatin appears to be typically two-fold denser than euchromatin, explaining the approximation of 2:1 for our calculations. To clarify the situation, we made the following modifications:
In the caption of Fig. 1b:

"Note that heterochromatin concentration variations are amplified using Hoechst because of its sequence preference for AT rich regions."

5. Suppl. 2b:
How is the observed variability of nucleoplasmic-to-cytoplasmic concentration of tracers to be explained?

The nucleoplasm is separated from the cytoplasm by the nuclear membrane. Nuclear pores are distributed on this membrane, and they regulate the flux of proteins between these two compartments. Size exclusion of the pore channel limits the passage of large inert molecules such as the mEGFP decamer, whereas mEGFP diffuses nearly freely through the pores. Hence, after their production in the cytoplasm, mEGFP decamers remain more concentrated in this compartment, leading to an apparent exclusion from the nucleus.
We clarified this point in the caption of Fig. S2:
"Cell-to-cell variability in the nucleoplasmic to cytoplasmic concentration ratio is observed, which is related to the fact that the passage of large tracers through nuclear pores is limited by the size exclusion of their central channel, thereby inducing an accumulation after translation in the cytoplasm."

6. Results Fig 5a and discussion p13 line 14
The behavior of RCC1 does not fit well with the fractal model. This is explained by a change of fractal dimension with time. Why does this not apply to the other two, H1.1 and H1-t?

From cell to cell, we observed some variability in the heterochromatin redistribution kinetics not only for RCC1, but also fro H1 and H1-t. The common feature of all our data is the delayed redistribution right after PA, which is extensively discussed in the paper as it is the main determinant of the fractal exponent h.
To clarify this point, we modified the text:
"In some cases for H1.1, RCC1 or H1t, the relaxation curves could not be fit with the fractal kinetics model at long time scales (upper cyan fit curve in Fig. 5a "RCC1"), suggesting that the fractal exponent ε may decrease with time."

7. Discussion: p. 15, second paragraph
Though diffusional kinetics to fill available space was identical for a given tracer everywhere in the nucleus (shown with PA-experiments), the residence times measured by FCS did increase with the crowding-status. Is this discrepancy of motional behavior determined by either PA or FCS attributable to the differences in the available volumes in euchromatin and heterochromatin, which were not corrected for in the calculation of the FCS-auto-correlation function in contrast to the adequate normalization of PA-experiments?

FCS measurements show a 2-3 fold diffusive hindrance in heterochromatin and nucleoli. The temporal and spatial resolution of PA in Fig. 2c, which are 200 ms and 1 m, respectively, are insufficient to detect such a small difference. Notably, using high speed photoactivation with line scanning confocal system, we observed a diffusion slow down in nucleoli in agreement with our FCS measurements (data not shown), therefore demonstrating that there is no conflict between the two techniques.
Second, the difference in concentration between heterochromatin and euchromatin is observed with FCS, and the absolute value of the auto-correlation function is proportional to the number of molecules in the confocal volume (Bacia, Methods, 2003). The shape of the ACF enables us to evaluate the mean residence time of protein in the confocal volume, independently of the number of molecules present in this volume.

8. Discussion p. 17/18
'...we predict, that heterochromatin exclusion relative to euchromatin should be 2 fold, in agreement with our measurement...'
The prediction precludes a 2-fold density-difference between eu- and heterochromatin. In contrast, according to Fig.1, measurements were based on 6-fold densitiy-ratios. Since

accessible volume fractions disproportionally decrease with increasing volume occupancies of crowders, the apparent agreement needs more explication.

This point is discussed above in our response to remark #4.

List of minor remarks:

- Results, p6 line 10: A very similar study was performed by Gorisch et al 2003 and should be cited together with Handwerger.

This omission has been rectified.

- Results p.6 line 11 and Fig. 1: The definition of 'exclusion' as the relative accessibility between two compartments is misleading, since tracers will become excluded from both compartments to some extent. 'Relative exclusion' may be more informative.

Excellent suggestion that has been taken into account in the remark #4.

- Results, p.7, line 10: 'Fig 4b' should read Fig 2b

This mistake has been amended.

- Suppl. 2b: A line-scan through the cells of the third lane could help to appreciate the relative differences of GFP-10 expression in the three examples.

This plot has been added to Supplementary Fig. 2.

- Suppl. S2c and d: The four tracer-sizes (mGFP through mGFP-10) in the four cumulated graphs should be distinguished, e.g. using color coding for the measure points as in Fig. 4b, to visualize the influence of tracer size separated from effects of the nucleoplasm (in c) and euchromatin (in d).

As suggested by the referee, we added colours to the graphs S2c, and S2d in order to improve the readability of the supplementary material. Note that we did not change the layout of Fig. S2d (left panel) because its readability is deteriorated with more information.

- Results p. 9, l.4 Enhancement of binding rates is a possible but not necessary consequence of crowding; it depends on the reaction (e.g. Minton 2001).

We adapted our statement on crowding predictions by mentioning:
"If crowding predictions apply in vivo, most reactions are expected to exhibit enhanced binding rates when their ligands are found in a crowded heterochromatin focus compared to less crowded euchromatin."

- Suppl. S3 a: 'triangles' are squares.

This mistake has been amended.

- Suppl. S3 c: How is the 'compensation for PAGFP photophysics' performed?

We clarified this point in the figure caption:
"... Note that we observe an increase in intensity right after whole nucleus photoactivation, which is an intrinsic photophysics property of PAGFP. The right plot represents heterochromatin curve compensated for PAGFP photophysics obtained by dividing the redistribution kinetics (blue dataset in the left graph) by the whole nucleus response (black dataset in the right graph)..."

- Suppl. S3 c: The plateau at short times scales is not readily appreciable, given the ups and downs throughout the entire curve progression. Could it be specified on the graph e.g. using arrows?

We added an arrowhead in this figure to highlight the plateau.

- Suppl. S3 d: Explain 'PDE'.

PDE stands for partial differential equations, and this term is defined in line 2 of the Supplementary Material just before their mathematical definition.

- Suppl. S3 e: The fit for heterochromatin (orange) not only is poor for short timescales. A smoothed plot of residuals probably would be more informative than the enlarged view of the short-time regime.

The plot has been changed according to the referee's suggestion.

-Suppl. S3 f: 'Heterochromatin dynamics...' should probably read like 'dynamics of tracers within heterochromatin'.

We changed the text following the referee's advice.

- Results p.9, lower part: 'Fig. 1c' is Fig. 3c.

This mistake has been corrected.

- Fig 4: labels d through f are out of scale.

This mistake has been corrected.

- p.15, first paragraph: 'We thus propose molecular crowding as force that promotes and stabilizes the self-organization of nuclear compartments.' This has been proposed by many others before (e.g. Herzfeld 1996, Marenduzzo et al. 2006, Richter et al. 2008) and should be taken into account with this statement.

We have cited these papers in the revised manuscript.

Referee #3

We are grateful to referee #3 that s/he highly recommends our work for publication. We fully agree with her/him on that our assays are not all original, but that their combination is a valuable achievement in the field. The interpretation of all our data

with a simple model based on a limited number of structural parameters provides a framework for future quantitative biological studies.

In their paper Bancaud et al. present a comprehensive approach to characterizing the effects of crowding due to chromatin organanization in vivo. They report several different experimental studies, i.e. PA, FCS and single particle tracking, to quantify the main effects related to crowding, i.e. excluded volume, slower diffusion and enhanced binding rates. Furthermore, they unify the results of these experiments in a single model in which chromatin organization is described in terms of fractal dimensions.

The experiments seem well designed for the tested models and performed at a high level. Though not all experiments seem original, it is definitely valuable that all were done in the same laboratory and where possible with the same cell lines and probes. The true value of this paper is in the integration of all methods and in the original way of testing and describing crowding effects in vivo. This is an important issue and has been relatively ignored before. That data and the interpretation in the results section appear sound though the use of the residence time for estimation of the size of the mEGFP probe may be less solid. If the FCS signal consists of a mixture of different species, this might also result in a broadened and shallower correlation curve. However, the anomaly parameter appears independent of size giving credit to this interpretation.

GFP multimers have been recently used in several rheological studies in the cell, e.g. (Pack et al., Biophys J, 2006) or (Dross et al., Plos One, 2009). This approach is conceptually attractive because monodisperse freely-diffusing tracers are directly expressed by the cells. However and to our surprise, the size of large GFP arrays appeared to be highly heterogeneous due to partial degradation (Fig. S2).
Our results based on steady-state nucleoplasmic-cytoplasmic intensity ratios and FCS show that the degree of degradation is highly heterogeneous from cell to cell (Fig. S2b), but they do not allow us to conclude on the degree of degradation in individual cells. Interestingly, the behavior of mEGFP and its dimer, which appear to be undegraded (Fig. S2), is anomalous and consistent with a fractal model, and this suggestion is strengthened by SPT and PA measurements.
As observed with mEGFP pentamer and decamer and expected from the fractal model, the rheological hindrance and the anomaly parameter are size-independent for all our probes, even if probes are partially degraded. Therefore, there is no contradiction with the fractal model in our measurements.
Finally, let us mention that our results are very similar to those published by (Pack et al., Biophys J, 2006), in which the FCS response of 5 GFP multimers is analyzed in details with a two-state model.

We added more information about the partial degradation in the caption of Suppl Fig. S2b:
"A large variability for high MW mEGFP arrays is thus observed in a cell population, but we can not conclude on the degree of degradation of these probes at the level of individual cells."

In my opinion the paper presents novel ideas and has the potential to have a large impact on the field. I can highly recommend this paper for publication.

Textual remarks:

Page 8, line 9 states a diffusion coefficient of 9.2 +/- 1.0 um2/s in euchromatin while in figure 2b it says 10.5

In the results section, we provide the mEGFP pentamer average diffusion coefficient with the standard deviation. In Fig. 2b, the plot represents the FCS data obtained in

one individual cell, from which we derive a diffusion coefficient of 10.5 um2/s. The latter value is expectedly close to the mean measurement.

Figure caption S5 e-f: remove repeated 'to chromatin'

This mistake has been corrected.

Figure caption S5 g: insert ... scale depends on 'the' tracking accuracy

This mistake has been corrected.

2nd Editorial Decision 19 October 2009

I have received the comments from one of the original referees who I asked to evaluate the revised version of the manuscript. I have to say that agree with this referee and feel disappointed that the opportunity was not taken to point out some of the potential limitations of your analysis. Nevertheless this referee does support publication of the study in the EMBO Journal. Your manuscript has been accepted and you will receive the official acceptance letter in the next day or so.

Referee #1 (Remarks to the Author):

I am not satisfied with the authors' response to my concerns. Changing "model" to "working model" is a minor revision. I was hoping instead that the authors would expand on why this is a working model, explaining some of the limitations and potential complications of their analysis. Despite this regret, I continue to think that the authors have introduced some important new ideas and so do not wish to hold up publication.

2nd Revision - authors' response 23 October 2009

Listed below are a few additional corrections that were made in the discussion section in order to address the referee's concerns on the fractal model.

Paragraph "Chromatin architecture is consistent with a fractal model, heterochromatin fills space more compactly than euchromatin":

a/ I quoted the latest Job Dekker paper that describes chromatin large scale fractal organization "through chromatin interminglement (Lieberman-Aiden et al., 2009)".

b/ I made a precision with respect to this Dekker paper:
"The fractal nature of chromatin has already been observed at small length scales based on neutron scattering of erythrocyte nuclei..."

c/ I stated that with we do not direct evidence for a fractal organization:
"Our live cell measurements do not provide a direct visualization of chromatin fractal architecture (visualizing chromatin topology under physiological conditions at high resolution is currently not possible by any method we are aware of), rather we obtain three independent lines of evidence that allow us to determine the fractal dimension of euchromatin to 2.2 and heterochromatin to 2.6."

d/ Conclusion of this paragraph:
"Although the model of a fractal organization of chromatin is consistent with all our observations, it certainly has limitations and will require future validations and refinements. For example using

photo-activated localization microscopy (Betzig et al., 2006) applied to cells expressing core histones tagged with photoswitchable fluorophores could provide structural information on chromatin with nanometer precision."

